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Biocontrol Activities of *Bacillus megaterium* Volatile Organic Compounds (VOCs) Against Mycotoxigenic *Aspergillus*, *Penicillium and Fusarium* fungi

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Abstract

Toxigenic fungi produce mycotoxins that are known to have adverse health effects. Biological control tools are one of the recent to be investigated.

In this project, our findings demonstrate that *Bacillus megaterium* and *Bacillus pumilus* produce volatile organic compounds (VOCs) that have antifungal activities controlling the growth and mycotoxin production of fungal species on maize. The bacterial volatiles identified includes nitropropane which is a known antifungal compound.

Objectives

1. Investigation of the in vitro antifungal activities of *B. megaterium* strain VOC.
2. Investigation of the reversibility of VOC effect on fungal growth and sporulation.
3. Effect of *B. megaterium* VOC on mycotoxins synthesis potential of the fungi.
4. Test for in-vivo fungal growth inhibition by bacterial VOC.
5. Identification of the VOC compounds produced by *B. megaterium* and *B. pumilus.*

Literature review

One of the known biological human health hazards are mycotoxins. Mycotoxins are toxic secondary metabolite chemicals produced by fungi (Richard, 2007). There are many types of mycotoxins including aflatoxins, fumonisin, and ochratoxins. Mycotoxins has negative effects on the human health and causes many diseases including Kwashiorkor, Reye's syndrome, balkan endemic nephropathy, Neural tube defects, and various types of cancers (Rychlik, 2017). Controlling fungi and mycotoxin production could be done by chemical, physical, thermal, and biological controls. Some bacterial species have antifungal activities either by volatile organic compounds (VOCs) or diffusible compounds (Zheng *et al.*, 2013) and diffusible (Bottone, 2003). *Bacillus megaterium* is a gram positive bacteria that survives in temperature between 3 °C and 45°C, with optimum temperature of 30°C (Boone *et al.*, 2001). In order to apply biocontrol methods in controlling fungi, further studies should be conducted on the nature of the soil, and the chemical, physical, and biological context that the bacteria will be in to ensure its ability to inhibit the fungi in that environment (Nguyen *et al.*, 2017).

Results and discussion

1. *B. megaterium* and *B. pumilus* VOC control the growth of fungi

Fig.1: Aspergillus flavus growth exposed to VOC of *B. megaterium* , *B. pumilus* and negative control, respectively.

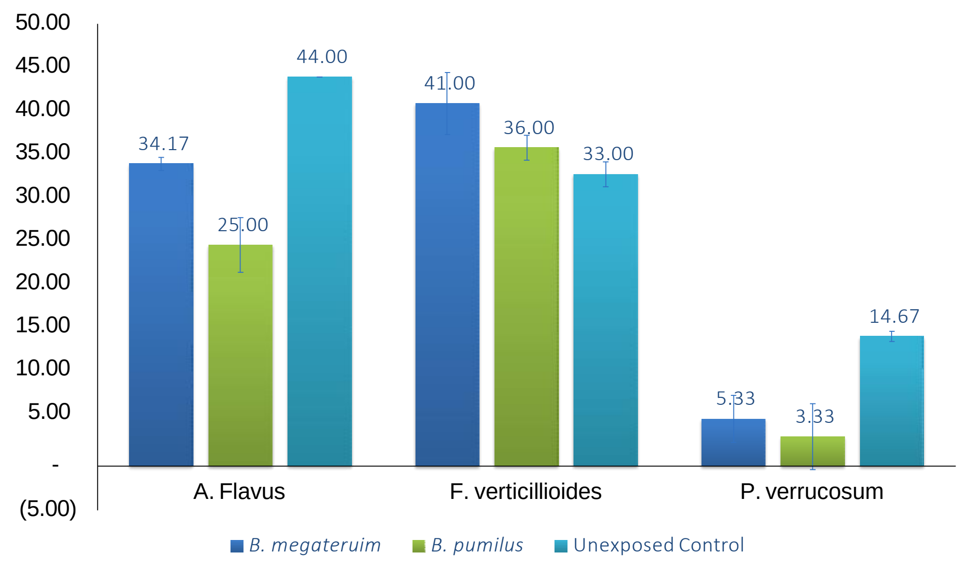

Fig.2: Fungal growth (mm diameter) under the effect of bacterial VOCs

- VOCs produced by *B. megaterium* controlled *A. flavus* growth by 22%, and P. verrucosum by 56%.
- VOCs produced by *B. Pumilus* controlled *A. flavus* growth by 49.6%, and *P. verrucosum* by 54.6%.

2. Effect of bacterial volatiles on fungi is reversible

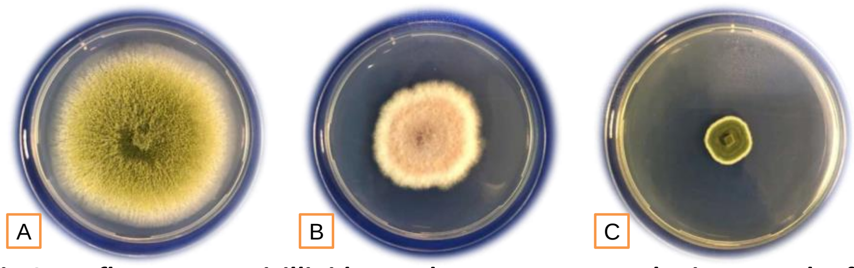

Fig.3: *A. flavus*, *F. verticillioides*, and *P. verrucosum* colonies growth after exposure to bacterial volatiles and re-inoculation on new media with no exposure.

- The effect of the bacterial volatiles is reversible and the fungi grows back normally when the VOCs exposure stops.

3. *B. megaterium* and *B. pumilus* are inhibiting the mycotoxin production of toxigenic fungi

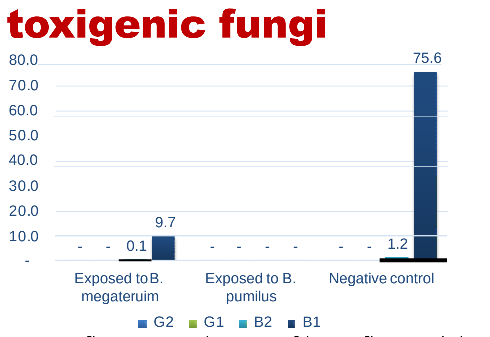

Fig. 4: Aflatoxin production of by *A. flavus* while exposed to bacterial VOCS (μg/Kg of media).

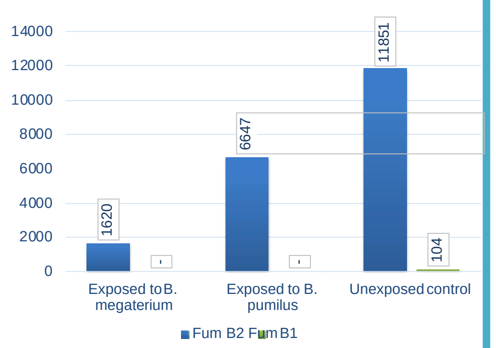

Fig. 5: Fumonisin production of by *F. verticillioides* while exposed to bacterial VOCS (ng*g/ml).

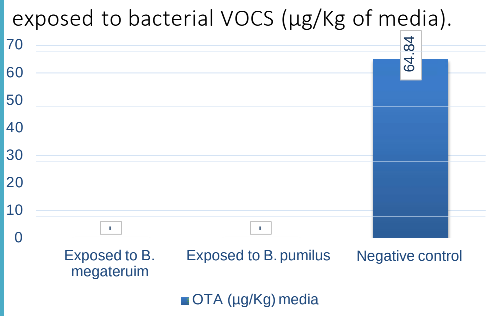

Fig. 6: ochratoxin production of by *P. verrucosum* while exposed to bacterial VOCS (μg/Kg of media).

B. megaterium and *B. pumilus* VOC inhibited the production of AF and FUM significantly and stopped OTA production completely.

4. Bacterial VOCs inhibit the growth of *A. flavus* on maize and inhibit the mycotoxin production significantly

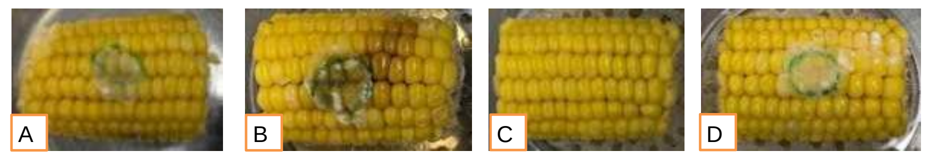

Fig. 7: Fungal growth on maize under the effect of bacterial VOCs. (A) represents *B. megaterium* with spore inoculation. (B) is *B. pumilus* with spore inoculation. (C) is control. (D) is negative control.

- ***B. megaterium* VOC controlled the growth of *A. flavus* on maize significantly.**
- **No significant inhibition was found by the *B. pumilus.***
- ***B. megaterium* and *B. pumilus* VOC have considerable inhibition on production of aflatoxins.**

5. *B. megaterium* and *B. pumilus* are producing antifungal VOCs by GC/MS-MS:

B. megaterium Day 1	Concen-tration
cis-1,2-Dichloroethene	0.77 μg/L
Methyl Acrylate	5.51 μg/L
2-Nitropropane	8.82 μg/L
Methylene Chloride	2.33 μg/L

Table 1. VOCs produced by *B. megaterium* – anoxic conditions.

B. megaterium Day 2	Concentr-ation
1,3-Dichloropropane	4.93 μg/L
Isobutanol	52.58 μg/L
2-Nitropropane	7.96 μg/L
Dibromofluorometha ne [ST]	60.34 μg/L

Table 2. VOCs produced by *B. megaterium* – aerobic conditions.

B. pumilus Day 1	Concent-ration
2-Nitropropane	9.32 μg/L
Acetonitrile	1.54 μg/L
Methylene Chloride	9.23 μg/L
Allyl Chloride	2.95 μg/L
Carbon Disulfide	2.95 μg/L

Table 3. VOCs produced by *B. Pumilus* – anoxic conditions.

B. pumilus Day 2	Concentra-tion
2-Nitropropane	7.33 μg/L
1,3-Dichloropropane	8.96 μg/L
Dibromofluorometha ne [ST]	55.54 μg/L
Isobutanol	108.64 μg/L

Table 4. VOCs produced by *B. pumilus* – aerobic conditions..

- **All treatments produce 2-Nitropropane.**
- **2-nitropropane concentration increases when bacteria is under oxidative stress.**

Conclusion

- Volatile organic compounds (VOCs) produced by *Bacillus megaterium* and *Bacillus pumilus* have antifungal activities.
- VOCs has various effects on the fungal growth inhibition.
- Effect of VOCs on fungal growth is reversible.
- Mycotoxin production is reduces significantly when fungi is exposed to *B. megaterium* and *B. pumilus* volatiles.
- Bacterial volatiles are identified including nitropropane known as antifungal compound.

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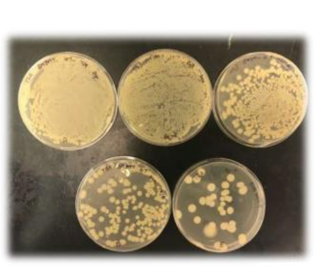
Materials & Methods

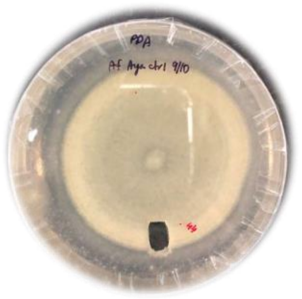
1. Investigation of the in vitro antifungal activities of *B. megaterium*:

A prepare 10^{-1} dilution of *Bacillus megaterium* and spread on TSA media, incubated 24 hours. Prepare fungal spore suspension. Transfer 4 μl of liquid spores into the center of PDA plates. Each fungi plate is sealed with a bacterial plate with parafilm and tape. Incubate plates at 26°C for 72 hours.



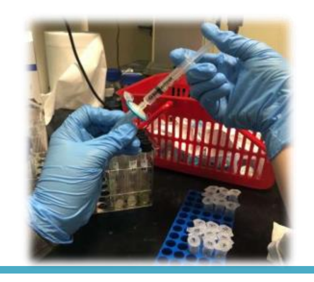
2. Investigation of the effectiveness of the VOCs on the fungi:

On day 7 of the incubation of fungi with bacteria, using a scalpel blade a cut from the fungi margin was taken and transferred to a new PDA plate. Incubated for 7 days at 26°C to check the fungal growth and operandum.



3. Determination of the concentrations of mycotoxins using HPLC:

Media plugs are taken by cork borer from experiment 1 and transferred to tubes. Mycotoxins extracted using appropriate solvents. Tubes are left in the Sonicator for 1 hour. Filtered using syringe filters. Analyzed by HPLC or LC-MS.

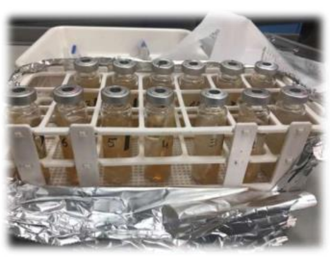


4. Testing the control on *Aspergillus flavus* on maize by VOCs produced by bacteria *in-vitro*:

Testing the effect of volatiles produced by both bacteria on the growth of *A. flavus*. Prepare LB broth for bacterial preculture and inoculate a loopful of *B. megaterium* and *B. pumilus* in saline solution. Incubate in 37.5°C for 18-24 hours. Prepare TSA media plates, spread bacteria on media. Incubate in 30°C for 24 hours. Prepare spores suspension (*Aspergillus flavus*). Full grown maize washed with bleach and distilled sterile water. Inoculate full grown maize with 10 μL of spore suspension. In order for the volatiles to reach the maize on top, holes are made into new sterile cover. Incubate in 26°C.

5. Identification of the VOCs produced by *B. megaterium* and *B. pumilus* using GC/MS-MS:

Prepare slant agar in Gas chromatography (GC) tubes. Spread bacteria on media. Incubate tubes for 6 days. Before starting the analysis, sonicate and then inject methanol with syringes into the tubes in order to dissolve and liquify the VOCs and becomes accessible to the GC machine.



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